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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/10, 1/20, 15/00, C07K 14/00, C07H 21/04, C12Q 1/48, A61K 39/395		A1	(11) International Publication Number: WO 99/67368 (43) International Publication Date: 29 December 1999 (29.12.99)
(21) International Application Number: PCT/US99/13683 (22) International Filing Date: 16 June 1999 (16.06.99) (30) Priority Data: 60/090,354 23 June 1998 (23.06.98) US 09/328,857 8 June 1999 (08.06.99) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventors: CASES, Sylvaine; Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100 (US). FARESE, Robert, V.; Gladstone Institute of Cardiovascular Disease, P.O. Box 419100, San Francisco, CA 94141-9100 (US). NOVAK, Sabine; 4th floor, Baaderstrasse 3, D-80469 Munich (DE). ERICKSON, Sandra, K.; University of California, San Francisco, School of Medicine, Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121 (US). (74) Agent: BERLINER, Robert; Fulbright & Jaworski L.L.P., 29th floor, 865 S. Figueroa Street, Los Angeles, CA 90017-2576 (US).			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NOVEL ACYL COA:CHOLESTEROL ACYLTRANSFERASE (ACAT-2)			
(57) Abstract <p>Nucleic acid compositions encoding novel ACAT proteins, as well as the novel ACAT-2 proteins, (ACAT-2) are provided. Also provided are methods of producing the subject nucleic acid and protein compositions. The subject polypeptide and nucleic acid compositions find use in a variety of applications, including research, diagnostic, and therapeutic agent screening applications, as well as in treatment therapies for disease conditions associated with ACAT-2 activity.</p>			

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NOVEL ACYL COA:CHOLESTEROL ACYLTRANSFERASE (ACAT-2)**ACKNOWLEDGMENT OF GOVERNMENT SUPPORT**

5 This invention was made with Government support under Grant Nos. R01 52069 and R01 57170 awarded by the National Institute of Health and support from the Veteran's Administration. The Government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

10 Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing date of the United States Provisional Patent Application Serial No. 60/090,354 filed June 23, 1998, the disclosure of which is herein incorporated by reference.

INTRODUCTION

15 **Field of the Invention**

 The field of the invention is enzymes, particularly acyltransferases.

Background of the Invention

 The ability to synthesize sterol esters is fundamental to most eukaryotic cells. Sterol esterification is thought to participate in the maintenance of cell membrane
20 sterols at levels optimal for normal cell function. In mammalian cells, cholesterol esterification is catalyzed by the enzyme acyl CoA:cholesterol acyltransferase (E.C. 2.3.1.26, ACAT). ACAT activity has been implicated in a number of physiologic processes. In the small intestine, ACAT has been proposed to play a role in cholesterol
absorption by maintaining a free cholesterol diffusion gradient across the enterocyte

surface through the intracellular formation of cholesterol esters. Cholesterol ester formation by ACAT has also been hypothesized to be important for the assembly and secretion of apolipoprotein B-containing lipoproteins in the intestine and the liver. In the adrenal glands and other steroidogenic tissues, ACAT synthesizes cholesterol esters that accumulate in cytosolic droplets where they can serve as cholesterol substrate stores for steroidogenesis. In macrophages, ACAT generates intracellular cholesterol esters that are stored as cytosolic lipid droplets, a characteristic feature of macrophage foam cells in atherosclerotic lesions.

Recent evidence has suggested that more than one ACAT exists in mammals. A human ACAT cDNA was first identified from a macrophage cDNA library. The disruption of the mouse homolog of this ACAT gene (*Acact*) yielded viable, ACAT-deficient (*Acact*^{-/-}) mice that were characterized by tissue-specific reductions in cholesterol esters. Cholesterol ester stores were markedly reduced in adrenal cortices and cultured peritoneal macrophages; however, substantial levels of ACAT activity were present in *Acact*^{-/-} livers, and intestinal cholesterol absorption was normal, indicating that another ACAT enzyme was active in these tissues. Studies examining the tissue distribution of *Acact* mRNA expression also supported the hypothesis that more than one ACAT exists, as did previous biochemical and ACAT inhibitor studies showing differences between liver and aorta/macrophage ACAT activities. The above results indicate that a second ACAT enzyme contributes to cholesterol esterification activity in the liver and small intestine.

As such, there is much interest in the identification, isolation and characterization of this putative second ACAT enzyme.

Relevant Literature

25 U.S. Patent No. 5,484,727 reports the cloning of the Human ACAT-1 gene.

Farese, "Acyl CoA:cholesterol acyltransferase genes and knockout mice," *Curr Opin Lipidol* (1998 Apr) 9(2):119-123, provides a review of the current knowledge of ACAT genes.

ACAT-1 is described in Goodman, D.S., *Physiol. Rev.* (1965) 45: 747-839; Suckling & Strange, *J. Lipids Res.* (1985) 26:647-671 and Chang et al., *Annu. Rev. Biochem.* (1997) 66: 613-638; Meiner et al., "Disruption of the acyl-CoA:cholesterol

- acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals," Proc Natl Acad Sci U S A (1996 Nov 26) 93(24):14041-14046; Meiner et al., "Tissue expression studies on the mouse acyl-CoA:cholesterol acyltransferase gene (Acact): findings supporting the existence of multiple cholesterol esterification enzymes in mice," J Lipid Res (1997 Sep) 38(9):1928-1933; Erickson et al., "Acyl-coenzyme A:cholesterol acyltransferase in human liver. In vitro detection and some characteristics of the enzyme," Metabolism (1980, Oct) 29(10):991-996; Tabas et al., "Acyl coenzyme A:cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate," J Biol Chem (1988 Jan 25) 263(3):1266-1272; and Uelmen et al., "Tissue-specific expression and cholesterol regulation of acylcoenzyme A:cholesterol acyltransferase (ACAT) in mice. Molecular cloning of mouse ACAT cDNA, chromosomal localization, and regulation of ACAT in vivo and in vitro," J Biol Chem (1995 Nov 3) 270(44):26192-26201.
- 15 The role of ACAT in various biological processes is discussed in: Field et al., Gastroenterology (1990) 99:539-551; Wilson et al., J. Lipid Res. (1994) 35:943-955; Dixon & Ginsberg, Annu. Rev. Biochem. (1993) 34:167-179; Brown & Goldstein, Annu. Rev. Biochem. (1983) 52:223-261.

20 SUMMARY OF THE INVENTION

- Nucleic acid compositions encoding novel polypeptide products with acyl CoA:cholesterol acyltransferase activity, as well as the polypeptide products (ACAT-2) encoded thereby and methods for producing the same, are provided. The subject polypeptide and nucleic acid compositions find use in a variety of applications, including research, diagnostic, and therapeutic agent screening applications, as well as in therapeutic applications.

DETAILED DESCRIPTION OF THE INVENTION

- Nucleic acid compositions encoding novel polypeptide products with acyl CoA:cholesterol acyltransferase activity, as well as the polypeptide products (ACAT-2) encoded thereby and methods for producing the same, are provided. The subject

polypeptide and/or nucleic acid compositions find use in a variety of different applications, including research, diagnostic, and therapeutic agent screening/ discovery/ preparation applications, as well as in the treatment disease conditions associated with ACAT-2 activity.

5

Before the subject invention is further described, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as variations of the particular embodiments may be made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended to be limiting. Instead, the scope of the present invention will be established by the appended claims.

In this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

20 NUCLEIC ACID COMPOSITIONS

Nucleic acid compositions encoding polypeptide products (hereinafter ACAT-2 and described in greater detail below), as well as fragments thereof, are provided. By nucleic acid composition is meant a composition comprising a sequence of DNA having an open reading frame that encodes an ACAT-2 polypeptide, i.e. a gene encoding a polypeptide having ACAT activity, where the encoded polypeptide is not ACAT-1 as disclosed in U.S. Patent No. 5,484,727, the disclosure of which is herein incorporated by reference, and is capable, under appropriate conditions, of being expressed as an ACAT-2 polypeptide. Also encompassed in this term are nucleic acids that are homologous, substantially similar or identical to the nucleic acids encoding ACAT-2 polypeptides or proteins. Thus, the subject invention provides genes

encoding mammalian ACAT-2, such as genes encoding human ACAT-2 and homologs thereof and mouse Acat-2 and homologs thereof.

The coding sequence of the mouse *Acat-2* gene, i.e. the mouse cDNA encoding the mouse Acat-2 enzyme, has the nucleic acid sequence identified as SEQ ID NO:01,

5 *infra. Acat-2* maps to mouse chromosome 15. The coding sequence of the human *ACAT-2* gene, i.e. the human cDNA encoding the human ACAT-2 enzyme, has the nucleic acid sequence identified as SEQ ID NO:02, *infra*. Of interest in many embodiments is a nucleic acid (or the complement thereof) that hybridizes with either SEQ ID NO.01 or SEQ ID NO. 01 under stringent conditions.

10 The source of homologous genes to those specifically listed above may be any mammalian species, e.g., primate species, particularly human; rodents, such as rats and mice, canines, felines, bovines, ovines, equines, yeast, nematodes, etc. Between mammalian species, e.g., human and mouse, homologs have substantial sequence similarity, e.g. at least 75% sequence identity, usually at least 90%, more usually at
15 least 95% between nucleotide sequences. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are
20 known in the art, such as BLAST, described in Altschul *et al.* (1990), *J. Mol. Biol.* 215:403-10, etc. Unless specified otherwise, all sequence identity values provided herein are determined using GCG (Genetics Computer Group, Wisconsin Package, Standard Settings, gap creation penalty 3.0, gap extension penalty 0.1). The sequences provided herein are essential for recognizing ACAT-2 related and homologous
25 polynucleotides in database searches.

Nucleic acids encoding the ACAT-2 proteins and ACAT-2 polypeptides of the subject invention may be cDNAs or genomic DNAs, as well as fragments thereof.

The term "*ACAT-2*- gene" shall be intended to mean the open reading frame encoding specific ACAT-2 proteins and polypeptides, and ACAT-2 introns, as well as adjacent

30 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 20 kb beyond the coding region, but possibly further in either direction. The

gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into a host genome.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns, when present, being removed by nuclear RNA splicing, to create a continuous open reading frame encoding an ACAT-2 protein.

A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, *etc.*, including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA may be isolated as a fragment of 100 kbp or smaller; and substantially free of flanking chromosomal sequence. The genomic DNA flanking the coding region, either 3' or 5', or internal regulatory sequences as sometimes found in introns, contains sequences required for proper tissue and stage specific expression.

The nucleic acid compositions of the subject invention may encode all or a part of the subject ACAT-2 proteins and polypeptides, described in greater detail *infra*. Double or single stranded fragments may be obtained from the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, DNA fragments will be at least 15 nt, usually at least 18 nt or 25 nt, and may be at least about 50 nt. Of interest in certain embodiments are fragments which encode the N terminal portion of the encoded ACAT-2 protein, as described in greater detail *infra*, where the N-terminal portion may be at least about the N-terminal 25, 50, 75, 80, 85, 90 or 95 residues. Of interest in other embodiments are fragments which encode the C-terminal portion of the ACAT-2 protein, where the C-terminal portion may be at least about the C-terminal 100, 200, 300, 400 or 410 residues. Also of interest are nucleic

acids in which the above N and C terminal encoding fragments flank an additional nucleic acid sequence, where this additional nucleic acid sequence may be anywhere from 10 to 200, usually from about 50 to 150 and more usually from about 50 to 100 nucleotides in length.

- 5 The *ACAT-2* genes of the subject invention are isolated and obtained in substantial purity, generally as other than an intact chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid sequences that do not include an *ACAT-2* sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more
10 nucleotides with which it is not normally associated on a naturally occurring chromosome.

In addition to the plurality of uses described in greater detail in following sections, the subject nucleic acid compositions find use in the preparation of all or a portion of the *ACAT-2* polypeptides, as described below.

15

POLYPEPTIDE COMPOSITIONS

- Also provided by the subject invention are polypeptides having *ACAT-2* activity, *i.e.* capable of catalyzing the esterification of cholesterol, as well as oxysterols, with fatty
20 acyl CoA substrates. In addition to being capable of catalyzing the esterification of cholesterol with a fatty acyl CoA substrates, the subject proteins are incapable of esterifying, at least to any substantial extent, the following substrates: ethanol, retinol, tocopherol, β -sitosterol, lanosterol, vitamins D1 and D2, or diacylglycerol. With respect to fatty acyl CoA substrates, the *ACAT-2* polypeptides exhibit the following preference:
25 palmitoyl \geq linoleoyl \geq oleoyl \geq arachidonyl. With *ACAT-2* polypeptides, linoleoyl and palmitoyl compete with oleoyl for incorporation into cholesterol esters, but arachidonyl competes less well. In certain *in vitro* assays (see those reported in the Experimental Section, *infra*), the subject *ACAT-2* polypeptides exhibit higher activity with oleoyl than with palmitoyl.

- 30 The term polypeptide composition as used herein refers to both the full length proteins as well as portions or fragments thereof. Also included in this term are

variations of the naturally occurring proteins, where such variations are homologous or substantially similar to the naturally occurring protein, as described in greater detail below, be the naturally occurring protein the human protein, mouse protein, or protein from some other species which naturally expresses an ACAT-2 enzyme, usually a mammalian species. In the following description of the subject invention, the term ACAT-2 is used to refer not only to the human form of the enzyme, but also to homologs thereof expressed in non-human species, e.g. murine, rat and other mammalian species.

The subject ACAT-2 proteins are, in their natural environment, trans-membrane proteins. The subject proteins are characterized by the presence of at least one potential tyrosine phosphorylation site present in the motif MK-X-H/Y-SF, and multiple hydrophobic domains, including 5 to 10, usually 6 to 9 hydrophobic domains capable of serving as trans-membrane regions. The proteins range in length from about 400 to 650, usually from about 475 to 525 and more usually from about 485 to 500 amino acid residues, and the projected molecular weight of the subject proteins based solely on the number of amino acid residues in the protein ranges from about 50 to 80, usually from about 55 to 75 and more usually from about 60 to 65 kDa, where the actual molecular weight may vary depending on the amount of glycosylation, if any, of the protein and the apparent molecular weight may be considerably less (40 to 50 kDa) due to SDS binding on gels.

The amino acid sequences of the subject proteins are characterized by having at least some homology to a corresponding ACAT-1 protein from the same species, e.g. a human ACAT-2 protein has at least some sequence homology with the human ACAT-1 protein, the mouse ACAT-2 protein has at least some sequence homology with the mouse Acat-1 protein, etc., where the sequence homology will not exceed about 80%, and usually will not exceed about 70% and more usually will not exceed about 60%, but will be at least about 30% and more usually at least about 40%, as determined using GCG (Genetics Computer Group, Wisconsin Package, Standard Settings, gap creation penalty 3.0, gap extension penalty 0.1).

Of particular interest in many embodiments are proteins that are non-naturally glycosylated. By non-naturally glycosylated is meant that the protein has a

glycosylation pattern, if present, which is not the same as the glycosylation pattern found in the corresponding naturally occurring protein. For example, human ACAT-2 of the subject invention and of this particular embodiment is characterized by having a glycosylation pattern, if it is glycosylated at all, that differs from that of naturally occurring human ACAT-2. Thus, the non-naturally glycosylated ACAT-2 proteins of this embodiment include non-glycosylated ACAT-2 proteins, i.e. proteins having no covalently bound glycosyl groups.

The activity of the ACAT-2 protein is inhibited by a number of different compounds, including non-specific inhibitors, such as PMSF, PHMB and progesterone, and specific inhibitors, such as PD 132301-2, CI-976 and CI-1011. ACAT-2 is more sensitive to CI-976 and CI-1011 than ACAT-1, having an IC_{50} value that is typically less than half that observed with ACAT-1 in the presence of the same inhibitor.

Of particular interest in certain embodiments is the mouse ACAT-2 protein, where the mouse ACAT-2 protein of the subject invention has an amino acid sequence that is substantially the same as or identical to the sequence appearing as SEQ ID NO:03 *infra*. By substantially the same as is meant a protein having a sequence that has at least about 80%, usually at least about 90% and more usually at least about 98% sequence identity with the sequence of SEQ ID NO:03, as measured by GCG, *supra*. Of particular interest are proteins encoded by a nucleic acid (or the complement thereof) that hybridizes to SEQ ID NO:01 under stringent conditions (defined *infra*).

Of particular interest in other embodiments is the human ACAT-2 protein, where the human ACAT-2 protein of the subject invention has an amino acid sequence that is substantially the same as, or identical to, the sequence appearing as SEQ ID NO:04, *infra*. Of particular interest are proteins encoded by a nucleic acid (or the complement thereof) that hybridizes to SEQ ID NO:02 under stringent conditions (defined *infra*).

In addition to the specific ACAT-2 proteins described above, homologs or proteins (or fragments thereof) from other species, i.e. other animal or plant species, are also provided, where such homologs or proteins may be from a variety of different types of species, usually mammals, e.g. rodents, such as mice, rats; domestic animals,

e.g. horse, cow, dog, cat; and humans. By homolog is meant a protein having at least about 35 %, usually at least about 40% and more usually at least about 60 % amino acid sequence identity the specific ACAT-2 proteins as identified in SEQ ID NOS: 04 to 06, where sequence identity is determined using the GCG, supra.

5 The ACAT-2 proteins of the subject invention (e.g. human ACAT-2, mouse ACAT-2 or homologs thereof) are present in a non-naturally occurring environment, e.g. are separated from their naturally occurring environment. In certain embodiments, the subject ACAT-2 protein is present in a composition that is enriched for ACAT-2 as compared to ACAT-2 in its naturally occurring environment. As such, purified ACAT-
10 2 is provided, where by purified is meant that ACAT-2 is present in a composition that is substantially free of non ACAT-2 proteins, where by substantially free is meant that less than 90 %, usually less than 60 % and more usually less than 50 % of the composition is made up of non-ACAT-2 proteins.

 In certain embodiments of interest, the ACAT-2 protein is present in a
15 composition that is substantially free of the constituents that are present in its naturally occurring environment. For example, a human ACAT-2 protein comprising composition according to the subject invention in this embodiment will be substantially, if not completely, free of those other biological constituents, such as proteins, carbohydrates, lipids, etc., with which it is present in its natural environment.
20 As such, protein compositions of these embodiments will necessarily differ from those that are prepared by purifying the protein from a naturally occurring source, where at least trace amounts of the protein's constituents will still be present in the composition prepared from the naturally occurring source.

 The ACAT-2 of the subject invention may also be present as an isolate, by
25 which is meant that the ACAT-2 is substantially free of both non-ACAT-2 proteins and other naturally occurring biologic molecules, such as oligosaccharides, polynucleotides and fragments thereof, and the like, where substantially free in this instance means that less than 70 %, usually less than 60% and more usually less than 50 % of the composition containing the isolated ACAT-2 is a non-ACAT-2 naturally
30 occurring biological molecule. In certain embodiments, the ACAT-2 is present in substantially pure form, where by substantially pure form is meant at least 95%,

usually at least 97% and more usually at least 99% pure.

In addition to the naturally occurring ACAT-2 proteins, ACAT-2 polypeptides which vary from the naturally occurring ACAT-2 proteins are also provided. By ACAT-2 polypeptides is meant proteins having an amino acid sequence encoded by an open reading frame (ORF) of an *ACAT-2* gene, described *supra*, including the full length ACAT-2 protein and fragments thereof, particularly biologically active fragments and/or fragments corresponding to functional domains; and including fusions of the subject polypeptides to other proteins or parts thereof. Fragments of interest will typically be at least about 10 aa in length, usually at least about 50 aa in length, and may be as long as 300 aa in length or longer, but will usually not exceed about 1000 aa in length, where the fragment will have a stretch of amino acids that is identical to an ACAT-2 protein of SEQ ID NO:03 or SEQ ID NO:04, or a homolog thereof; of at least about 10 aa, and usually at least about 15 aa, and in many embodiments at least about 50 aa in length. In certain embodiments, N-terminal fragments of the ACAT-2 protein are of interest, e.g. fragments of the N-terminal 25, 50, 75, 80, 85, 90 or 95 residues. Of interest in other embodiments are fragments the C-terminal portion of the ACAT-2 protein, where the C-terminal portion may be at least about the C-terminal 100, 200, 300, 400 or 410 residues. Also of interest are fusion proteins of at least one of, or both of, the above C and N terminal fragments with an additional polypeptide sequence. In certain embodiments, of interest is a fusion protein in which the above N and C terminal fragments flank an additional sequence, where this additional sequence may be anywhere from about 3 to 75, usually from about 10 to 50 and more usually from about 10 to 30 residues in length.

25

PREPARATION OF ACAT-2 POLYPEPTIDES

The subject ACAT-2 proteins and polypeptides may be obtained from naturally occurring sources, but are preferably synthetically produced. Where obtained from naturally occurring sources, the source chosen will generally depend on the species from which the ACAT-2 is to be derived.

30

The subject ACAT-2 polypeptide compositions may be synthetically derived by expressing a recombinant gene encoding ACAT-2, such as the polynucleotide compositions described above, in a suitable host. For expression, an expression cassette may be employed. The expression vector will provide a transcriptional and translational initiation region, which may be inducible or constitutive, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region. These control regions may be native to an *ACAT-2* gene, or may be derived from exogenous sources.

Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences encoding heterologous proteins. A selectable marker operative in the expression host may be present. Expression vectors may be used for the production of fusion proteins, where the exogenous fusion peptide provides additional functionality, i.e. increased protein synthesis, stability, reactivity with defined antisera, an enzyme marker, e.g. -galactosidase, etc.

Expression cassettes may be prepared comprising a transcription initiation region, the gene or fragment thereof, and a transcriptional termination region. Of particular interest is the use of sequences that allow for the expression of functional epitopes or domains, usually at least about 8 amino acids in length, more usually at least about 15 amino acids in length, to about 25 amino acids, and up to the complete open reading frame of the gene. After introduction of the DNA, the cells containing the construct may be selected by means of a selectable marker, the cells expanded and then used for expression.

ACAT-2 proteins and polypeptides may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production of the protein, a unicellular organism, such as *E. coli*, *B. subtilis*, *S. cerevisiae*, insect cells in combination with baculovirus vectors, or cells of a higher organism such as vertebrates, particularly mammals, e.g. COS 7 cells, may be used as the expression host cells. In some situations, it is desirable to express the *ACAT-2* gene in eukaryotic cells, where the ACAT-2 protein will benefit

from native folding and post-translational modifications. Small peptides can also be synthesized in the laboratory. Polypeptides that are subsets of the complete ACAT-2 sequence may be used to identify and investigate parts of the protein important for function.

- 5 Once the source of the protein is identified and/or prepared, e.g. a transfected host expressing the protein is prepared, the protein is then purified to produce the desired ACAT-2 comprising composition. Any convenient protein purification procedures may be employed, where suitable protein purification methodologies are described in Guide to Protein Purification, (Deuthser ed.) (Academic Press, 1990).
- 10 For example, a lysate may be prepared from the original source, e.g. naturally occurring cells or tissues that express ACAT-2 or the expression host expressing ACAT-2, and purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, and the like.

15 USES OF THE SUBJECT ACAT-2 POLYPEPTIDE AND NUCLEIC ACID COMPOSITIONS

- The subject polypeptide and nucleic acid compositions find use in a variety of different applications, including research, diagnostic, and therapeutic agent screening/discovery/preparation applications, as well as the treatment of disease
- 20 conditions associated with ACAT-2 activity.

RESEARCH APPLICATIONS

- The subject nucleic acid compositions find use in a variety of research
- 25 applications. Research applications of interest include: (a) the identification of *ACAT-2* homologs; (b) as a source of novel promoter elements; (c) the identification of ACAT-2 expression regulatory factors; (d) as probes and primers in hybridization applications, e.g. PCR; (e) the identification of expression patterns in biological specimens; (f) the preparation of cell or animal models for ACAT-2 function; (g) the
- 30 preparation of *in vitro* models for ACAT-2 function; etc.

Identification of *ACAT-2* homologs

Homologs of *ACAT-2* are identified by any of a number of methods. A fragment of the provided cDNA may be used as a hybridization probe against a cDNA library from the target organism of interest, where low stringency conditions are used. The probe may be a large fragment, or one or more short degenerate primers. Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 6×SSC (0.9 M sodium chloride/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1×SSC (0.15 M sodium chloride/0.015 M sodium citrate). Sequence identity may be determined by hybridization under stringent conditions, for example, at 50°C or higher and 0.1×SSC (15 mM sodium chloride/0.15 mM sodium citrate). Nucleic acids having a region of substantial identity to the provided *ACAT-2* sequences, e.g. allelic variants, genetically altered versions of the gene, etc., bind to the provided *ACAT-2* sequences under stringent hybridization conditions. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes.

Identification of Novel Promoter Elements

The sequence of the 5' flanking region may be utilized for promoter elements, including enhancer binding sites, that provide for regulation in tissues where *ACAT-2* is expressed. The tissue specific expression is useful for determining the pattern of expression, and for providing promoters that mimic the native pattern of expression. Naturally occurring polymorphisms in the promoter region are useful for determining natural variations in expression, particularly those that may be associated with disease.

Identification of *ACAT-2* Expression Regulatory Factors

Alternatively, mutations may be introduced into the promoter region to determine the effect of altering expression in experimentally defined systems. Methods for the identification of specific DNA motifs involved in the binding of

transcriptional factors are known in the art, *e.g.* sequence similarity to known binding motifs, gel retardation studies, *etc.* For examples, see Blackwell *et al.* (1995), *Mol. Med.* 1:194-205; Mortlock *et al.* (1996), *Genome Res.* 6:327-33; and Joulin and Richard-Foy (1995), *Eur. J. Biochem.* 232:620-626.

- 5 The regulatory sequences may be used to identify *cis* acting sequences required for transcriptional or translational regulation of *ACAT-2* expression, especially in different tissues or stages of development, and to identify *cis* acting sequences and *trans*-acting factors that regulate or mediate *ACAT-2* expression. Such transcription or translational control regions may be operably linked to an *ACAT-2* gene in order to
- 10 promote expression of wild type or altered *ACAT-2* or other proteins of interest in cultured cells, or in embryonic, fetal or adult tissues, and for gene therapy.

Probes and Primers

- 15 Small DNA fragments are useful as primers for PCR, hybridization screening probes, *etc.* Larger DNA fragments, *i.e.* greater than 100 nt are useful for production of the encoded polypeptide, as described in the previous section. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most
- 20 applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to choose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to
- 25 complementary strands of DNA, and will prime towards each other.

Identification of Expression Patterns in Biological Specimens

- The DNA may also be used to identify expression of the gene in a biological
- 30 specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well established in the literature.

Briefly, DNA or mRNA is isolated from a cell sample. The mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by polymerase chain reaction amplification using primers specific for the subject DNA sequences. Alternatively, the mRNA sample is separated by gel
5 electrophoresis, transferred to a suitable support, *e.g.* nitrocellulose, nylon, *etc.*, and then probed with a fragment of the subject DNA as a probe. Other techniques, such as oligonucleotide ligation assays, *in situ* hybridizations, and hybridization to DNA probes arrayed on a solid chip may also find use. Detection of mRNA hybridizing to the subject sequence is indicative of *ACAT-2* gene expression in the sample.

10

The Preparation of *ACAT-2* Mutants

The sequence of an *ACAT-2* gene, including flanking promoter regions and coding regions, may be mutated in various ways known in the art to generate targeted changes in promoter strength, sequence of the encoded protein, *etc.* The DNA
15 sequence or protein product of such a mutation will usually be substantially similar to the sequences provided herein, *i.e.* will differ by at least one nucleotide or amino acid, respectively, and may differ by at least two but not more than about ten nucleotides or amino acids. The sequence changes may be substitutions, insertions, deletions, or a combination thereof. Deletions may further include larger changes, such as deletions
20 of a domain or exon. Other modifications of interest include epitope tagging, *e.g.* with the FLAG system, HA, *etc.* For studies of subcellular localization, fusion proteins with green fluorescent proteins (GFP) may be used.

Techniques for *in vitro* mutagenesis of cloned genes are known. Examples of protocols for site specific mutagenesis may be found in Gustin *et al.* (1993),
25 *Biotechniques* 14:22; Barany (1985), *Gene* 37:111-23; Colicelli *et al.* (1985), *Mol. Gen. Genet.* 199:537-9; and Prentki *et al.* (1984), *Gene* 29:303-13. Methods for site specific mutagenesis can be found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, CSH Press 1989, pp. 15.3-15.108; Weiner *et al.* (1993), *Gene* 126:35-41; Sayers *et al.* (1992), *Biotechniques* 13:592-6; Jones and Winistorfer
30 (1992), *Biotechniques* 12:528-30; Barton *et al.* (1990), *Nucleic Acids Res* 18:7349-55; Marotti and Tomich (1989), *Gene Anal. Tech.* 6:67-70; and Zhu (1989), *Anal Biochem*

177:120-4. Such mutated genes may be used to study structure-function relationships of ACAT-2, or to alter properties of the protein that affect its function or regulation.

Production of *In Vivo* Models of ACAT-2 Function

5

The subject nucleic acids can be used to generate transgenic, non-human animals or site specific gene modifications in cell lines. Transgenic animals may be made through homologous recombination, where the normal *Acat-2* locus is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome.

10 Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like.

The modified cells or animals are useful in the study of *ACAT-2* function and regulation. For example, a series of small deletions and/or substitutions may be made in the host's native *ACAT-2* gene to determine the role of different exons in cholesterol metabolism, e.g. cholesterol ester synthesis, cholesterol absorption, *etc.* Specific constructs of interest include anti-sense *ACAT-2*, which will block *ACAT-2* expression, expression of dominant negative *ACAT-2* mutations, and over-expression of *ACAT-2* genes. Where an *ACAT-2* sequence is introduced, the introduced sequence may be either a complete or partial sequence of an *ACAT-2* gene native to the host, or
15 may be a complete or partial *ACAT-2* sequence that is exogenous to the host animal, e.g., a human *ACAT-2* sequence. A detectable marker, such as *lac Z*, may be introduced into the *acat-2* locus, where upregulation of *ACAT-2* expression will result in an easily detected change in phenotype.

One may also provide for expression of the *ACAT-2* gene or variants thereof in
25 cells or tissues where it is not normally expressed, at levels not normally present in such cells or tissues, or at abnormal times of development.

DNA constructs for homologous recombination will comprise at least a portion of the *ACAT-2* gene native to the species of the host animal, wherein the gene has the desired genetic modification(s), and includes regions of homology to the target locus.
30 DNA constructs for random integration need not include regions of homology to mediate recombination. Conveniently, markers for positive and negative selection are

included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown *et al.* (1990), *Meth. Enzymol.* **185**:527-537.

For embryonic stem (ES) cells, an ES cell line may be employed, or embryonic
5 cells may be obtained freshly from a host, *e.g.* mouse, rat, guinea pig, *etc.* Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF). When ES or embryonic cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct
10 may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized,
15 and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting offspring screened for the construct. By providing for a different phenotype of the blastocyst and the genetically modified cells, chimeric progeny can be readily detected.

20 The chimeric animals are screened for the presence of the modified gene and males and females having the modification are mated to produce homozygous progeny. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogeneic or congenic grafts or transplants, or in *in vitro* culture. The transgenic animals may be any non-human mammal, such as
25 laboratory animals, domestic animals, *etc.* The transgenic animals may be used in functional studies, drug screening, *etc.*, *e.g.* to determine the effect of a candidate drug on ACAT-2 activity.

Production of *In Vitro* Models of ACAT-2 Function

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One can also use the polypeptide compositions of the subject invention to

produce *in vitro* models of ACAT-2 function, e.g. the ability to catalyze the esterification of cholesterol with a fatty acyl CoA substrate. Such models will generally at least include the subject ACAT-2 proteins and ACAT-2 substrates, such as cholesterol and fatty acyl CoAs.

5

DIAGNOSTIC APPLICATIONS

Also provided are methods of diagnosing disease states associated with ACAT-2 activity, e.g. based on observed levels of ACAT-2 or the expression level of the ACAT-2 gene in a biological sample of interest. Samples, as used herein, include biological fluids such as semen, blood, cerebrospinal fluid, tears, saliva, lymph, dialysis fluid and the like; organ or tissue culture derived fluids; and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids. The cells may be dissociated, in the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

A number of methods are available for determining the expression level of a gene or protein in a particular sample. Diagnosis may be performed by a number of methods to determine the absence or presence or altered amounts of normal or abnormal ACAT-2 in a patient sample. For example, detection may utilize staining of cells or histological sections with labeled antibodies, performed in accordance with conventional methods. Cells are permeabilized to stain cytoplasmic molecules. The antibodies of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemilumescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Alternatively, the secondary antibody conjugated to a fluorescent compound, e.g. fluorescein, rhodamine, Texas red, etc. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by

various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, *etc.*

Alternatively, one may focus on the expression of *ACAT-2*. Biochemical studies may be performed to determine whether a sequence polymorphism in an
5 *ACAT-2* coding region or control regions is associated with disease. Disease associated polymorphisms may include deletion or truncation of the gene, mutations that alter expression level, that affect the activity of the protein, *etc.*

Changes in the promoter or enhancer sequence that may affect expression levels of *ACAT-2* can be compared to expression levels of the normal allele by various
10 methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed natural protein; insertion of the variant control element into a vector with a reporter gene such as β -galactosidase, luciferase, chloramphenicol acetyltransferase, *etc.* that provides for convenient quantitation; and the like.

15 A number of methods are available for analyzing nucleic acids for the presence of a specific sequence, *e.g.* a disease associated polymorphism. Where large amounts of DNA are available, genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis. Cells that express *ACAT-2* may be used as a source of mRNA, which may be assayed
20 directly or reverse transcribed into cDNA for analysis. The nucleic acid may be amplified by conventional techniques, such as the polymerase chain reaction (PCR), to provide sufficient amounts for analysis. The use of the polymerase chain reaction is described in Saiki, *et al.* (1985), *Science* 239:487, and a review of techniques may be found in Sambrook, *et al.* Molecular Cloning: A Laboratory Manual, CSH Press 1989,
25 pp.14.2–14.33. Alternatively, various methods are known in the art that utilize oligonucleotide ligation as a means of detecting polymorphisms, for examples see Riley *et al.* (1990), *Nucl. Acids Res.* 18:2887-2890; and Delahunty *et al.* (1996), *Am. J. Hum. Genet.* 58:1239-1246.

A detectable label may be included in an amplification reaction. Suitable
30 labels include fluorochromes, *e.g.* fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM),

- 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, *e.g.* ^{32}P , ^{35}S , ^3H ; *etc.* The label may be a two stage system, where the amplified
- 5 DNA is conjugated to biotin, haptens, *etc.* having a high affinity binding partner, *e.g.* avidin, specific antibodies, *etc.*, where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.
- 10 The sample nucleic acid, *e.g.* amplified or cloned fragment, is analyzed by one of a number of methods known in the art. The nucleic acid may be sequenced by dideoxy or other methods, and the sequence of bases compared to a wild-type *ACAT-2* sequence. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, *etc.* The hybridization pattern of a control and
- 15 variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in US 5,445,934, or in WO 95/35505, may also be used as a means of detecting the presence of variant sequences. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices are used to detect conformational changes
- 20 created by DNA sequence variation as alterations in electrophoretic mobility. Alternatively, where a polymorphism creates or destroys a recognition site for a restriction endonuclease, the sample is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel or capillary electrophoresis, particularly acrylamide
- 25 or agarose gels.
- Screening for mutations in *ACAT-2* may be based on the functional or antigenic characteristics of the protein. Protein truncation assays are useful in detecting deletions that may affect the biological activity of the protein. Various immunoassays designed to detect polymorphisms in *ACAT-2* proteins may be used in
- 30 screening. Where many diverse genetic mutations lead to a particular disease phenotype, functional protein assays have proven to be effective screening tools. The

activity of the encoded ACAT-2 protein may be determined by comparison with the wild-type protein.

Diagnostic methods of the subject invention in which the level of *ACAT-2* expression is of interest will typically involve comparison of the *ACAT-2* nucleic acid abundance of a sample of interest with that of a control value to determine any relative differences, where the difference may be measured qualitatively and/or quantitatively, which differences are then related to the presence or absence of an abnormal *ACAT-2* expression pattern. A variety of different methods for determining the nucleic acid abundance in a sample are known to those of skill in the art, where particular methods of interest include those described in: Pietu et al., *Genome Res.* (June 1996) 6: 492-503; Zhao et al., *Gene* (April 24, 1995) 156: 207-213; Soares, *Curr. Opin. Biotechnol.* (October 1997) 8: 542-546; Raval, J. *Pharmacol Toxicol Methods* (November 1994) 32: 125-127; Chalifour et al., *Anal. Biochem* (February 1, 1994) 216: 299-304; Stolz & Tuan, *Mol. Biotechnol.* (December 1996) 6: 225-230; Hong et al., *Bioscience Reports* (1982) 2: 907; and McGraw, *Anal. Biochem.* (1984) 143: 298. Also of interest are the methods disclosed in WO 97/27317, the disclosure of which is herein incorporated by reference.

SCREENING ASSAYS

20

The subject ACAT-2 polypeptides find use in various screening assays designed to identify therapeutic agents. The screening methods will typically be assays which provide for qualitative/quantitative measurements of enzyme activity in the presence of a particular candidate therapeutic agent. For example, the assay could be an assay which measures the esterification activity of ACAT-2 in the presence and absence of a candidate inhibitor agent. The screening method may be an *in vitro* or *in vivo* format, where both formats are readily developed by those of skill in the art. Depending on the particular method, one or more of, usually one of, the components of the screening assay may be labeled, where by labeled is meant that the components comprise a detectable moiety, *e.g.* a fluorescent or radioactive tag, or a member of a signal producing system, *e.g.* biotin for binding to an enzyme-streptavidin conjugate in

30

which the enzyme is capable of converting a substrate to a chromogenic product.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used.

A variety of different candidate agents may be screened by the above methods. Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

ACAT-2 NUCLEIC ACID AND POLYPEPTIDE THERAPEUTIC COMPOSITIONS

The nucleic acid compositions of the subject invention also find use as therapeutic agents in situations where one wishes to enhance ACAT-2 activity in a host, e.g. in a mammalian host in which ACAT-2 activity is sufficiently low such that a disease condition is present, etc. The *ACAT-2* genes, gene fragments, or the encoded

5 ACAT-2 protein or protein fragments are useful in gene therapy to treat disorders associated with *ACAT-2* defects. Expression vectors may be used to introduce the *ACAT-2* gene into a cell. Such vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription

10 initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, e.g. plasmid; retrovirus, e.g. lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

15 The gene or ACAT-2 protein may be introduced into tissues or host cells by any number of routes, including viral infection, microinjection, or fusion of vesicles. Jet injection may also be used for intramuscular administration, as described by Furth *et al.* (1992), *Anal Biochem* 205:365-368. The DNA may be coated onto gold microparticles, and delivered intradermally by a particle bombardment device, or

20 "gene gun" as described in the literature (see, for example, Tang *et al.* (1992), *Nature* 356:152-154), where gold microprojectiles are coated with the DNA, then bombarded into skin cells.

METHODS OF MODULATING ACAT-2 ACTIVITY IN A HOST

25

Also provided are methods of regulating, including enhancing and inhibiting, ACAT-2 activity in a host. Where the ACAT-2 activity occurs *in vivo* in a host, an effective amount of active agent that modulates the activity, e.g. reduces the activity, of ACAT-2 *in vivo*, is administered to the host. The active agent may be a variety of

30 different compounds, including a naturally occurring or synthetic small molecule compound, an antibody, fragment or derivative thereof, an antisense composition, and

the like.

Naturally occurring or synthetic small molecule compounds of interest include numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than
5 about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures
10 substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Also of interest as active agent are antibodies that modulate, e.g. reduce, if not inhibit, ACAT-2 activity in the host. Suitable antibodies are obtained by immunizing a
15 host animal with peptides comprising all or a portion of an ACAT-2 protein, such as found in the ACAT-2 polypeptide compositions of the subject invention. Suitable host animals include mouse, rat sheep, goat, hamster, rabbit, *etc.* The origin of the protein immunogen may be mouse, human, rat, monkey *etc.* The host animal will generally be a different species than the immunogen, e.g. human ACAT-2 used to immunize mice,
20 *etc.*

The immunogen may comprise the complete protein, or fragments and derivatives thereof. Preferred immunogens comprise all or a part of ACAT-2, where these residues contain the post-translation modifications, such as glycosylation, found on the native ACAT-2. Immunogens comprising the extracellular domain are
25 produced in a variety of ways known in the art, e.g. expression of cloned genes using conventional recombinant methods, isolation from HEC, *etc.*

For preparation of polyclonal antibodies, the first step is immunization of the host animal with ACAT-2, where the ACAT-2 will preferably be in substantially pure form, comprising less than about 1% contaminant. The immunogen may comprise
30 complete ACAT-2, fragments or derivatives thereof. To increase the immune response of the host animal, the ACAT-2 may be combined with an adjuvant, where suitable

adjuvants include alum, dextran, sulfate, large polymeric anions, oil& water emulsions, e.g. Freund's adjuvant, Freund's complete adjuvant, and the like. The ACAT-2 may also be conjugated to synthetic carrier proteins or synthetic antigens. A variety of hosts may be immunized to produce the polyclonal antibodies. Such hosts
5 include rabbits, guinea pigs, rodents, e.g. mice, rats, sheep, goats, and the like. The ACAT-2 is administered to the host, usually intradermally, with an initial dosage followed by one or more, usually at least two, additional booster dosages. Following immunization, the blood from the host will be collected, followed by separation of the serum from the blood cells. The Ig present in the resultant antiserum may be further
10 fractionated using known methods, such as ammonium salt fractionation, DEAE chromatography, and the like.

Monoclonal antibodies are produced by conventional techniques. Generally, the spleen and/or lymph nodes of an immunized host animal provide a source of plasma cells. The plasma cells are immortalized by fusion with myeloma cells to
15 produce hybridoma cells. Culture supernatant from individual hybridomas is screened using standard techniques to identify those producing antibodies with the desired specificity. Suitable animals for production of monoclonal antibodies to the human protein include mouse, rat, hamster, *etc.* To raise antibodies against the mouse protein, the animal will generally be a hamster, guinea pig, rabbit, *etc.* The antibody
20 may be purified from the hybridoma cell supernatants or ascites fluid by conventional techniques, *e.g.* affinity chromatography using ACAT-2 bound to an insoluble support, protein A sepharose, *etc.*

The antibody may be produced as a single chain, instead of the normal multimeric structure. Single chain antibodies are described in Jost *et al.* (1994) J.B.C.
25 269:26267-73, and others. DNA sequences encoding the variable region of the heavy chain and the variable region of the light chain are ligated to a spacer encoding at least about 4 amino acids of small neutral amino acids, including glycine and/or serine. The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody.

30 For *in vivo* use, particularly for injection into humans, it is desirable to decrease the antigenicity of the antibody. An immune response of a recipient against

the blocking agent will potentially decrease the period of time that the therapy is effective. Methods of humanizing antibodies are known in the art. The humanized antibody may be the product of an animal having transgenic human immunoglobulin constant region genes (see for example International Patent Applications WO 90/10077 and WO 90/04036). Alternatively, the antibody of interest may be engineered by recombinant DNA techniques to substitute the CH1, CH2, CH3, hinge domains, and/or the framework domain with the corresponding human sequence (see WO 92/02190).

The use of Ig cDNA for construction of chimeric immunoglobulin genes is known in the art (Liu *et al.* (1987) P.N.A.S. **84**:3439 and (1987) J. Immunol. **139**:3521). mRNA is isolated from a hybridoma or other cell producing the antibody and used to produce cDNA. The cDNA of interest may be amplified by the polymerase chain reaction using specific primers (U.S. Patent nos. 4,683,195 and 4,683,202). Alternatively, a library is made and screened to isolate the sequence of interest. The DNA sequence encoding the variable region of the antibody is then fused to human constant region sequences. The sequences of human constant regions genes may be found in Kabat *et al.* (1991) Sequences of Proteins of Immunological Interest, N.I.H. publication no. 91-3242. Human C region genes are readily available from known clones. The choice of isotype will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity. Preferred isotypes are IgG1, IgG3 and IgG4. Either of the human light chain constant regions, kappa or lambda, may be used. The chimeric, humanized antibody is then expressed by conventional methods.

Antibody fragments, such as Fv, F(ab')₂ and Fab may be prepared by cleavage of the intact protein, *e.g.* by protease or chemical cleavage. Alternatively, a truncated gene is designed. For example, a chimeric gene encoding a portion of the F(ab')₂ fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Consensus sequences of H and L J regions may be used to design oligonucleotides for use as primers to introduce useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments. C region

cDNA can be modified by site directed mutagenesis to place a restriction site at the analogous position in the human sequence.

Expression vectors include plasmids, retroviruses, YACs, EBV derived episomes, and the like. A convenient vector is one that encodes a functionally complete human CH or CL immunoglobulin sequence, with appropriate restriction sites engineered so that any VH or VL sequence can be easily inserted and expressed. In such vectors, splicing usually occurs between the splice donor site in the inserted J region and the splice acceptor site preceding the human C region, and also at the splice regions that occur within the human CH exons. Polyadenylation and transcription termination occur at native chromosomal sites downstream of the coding regions. The resulting chimeric antibody may be joined to any strong promoter, including retroviral LTRs, *e.g.* SV-40 early promoter, (Okayama *et al.* (1983) Mol. Cell. Bio. 3:280), Rous sarcoma virus LTR (Gorman *et al.* (1982) P.N.A.S. 79:6777), and moloney murine leukemia virus LTR (Grosschedl *et al.* (1985) Cell 41:885); native Ig promoters, *etc.*

In yet other embodiments of the invention, the active agent is an agent that modulates, and generally decreases or down regulates, the expression of *ACAT-2* in the host. Antisense molecules can be used to down-regulate expression of *ACAT-2* in cells. The anti-sense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, *e.g.* by reducing the amount of mRNA available for translation, through activation of RNase H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20

nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in
5 length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996), *Nature Biotechnol.* 14:840-844).

A specific region or regions of the endogenous sense strand mRNA sequence is chosen to be complemented by the antisense sequence. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several
10 candidate sequences are assayed for inhibition of expression of the target gene in an *in vitro* or animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1993), *supra*, and Milligan *et al.*, *supra*.) Preferred
15 oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

Among useful changes in the backbone chemistry are phosphorothioates;
20 phosphorodithioates, where both of the non-bridging oxygens are substituted with sulfur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH₂-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire ribose phosphodiester backbone with a peptide
25 linkage. Sugar modifications are also used to enhance stability and affinity. The α -anomer of deoxyribose may be used, where the base is inverted with respect to the natural β -anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity. Modification of the heterocyclic bases must maintain proper base pairing.
30 Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-

deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

As an alternative to anti-sense inhibitors, catalytic nucleic acid compounds,
5 *e.g.* ribozymes, anti-sense conjugates, *etc.* may be used to inhibit gene expression.
Ribozymes may be synthesized *in vitro* and administered to the patient, or may be encoded on an expression vector, from which the ribozyme is synthesized in the targeted cell (for example, see International patent application WO 9523225, and Beigelman *et al.* (1995), *Nucl. Acids Res.* 23:4434-42). Examples of oligonucleotides
10 with catalytic activity are described in WO 9506764. Conjugates of anti-sense ODN with a metal complex, *e.g.* terpyridylCu(II), capable of mediating mRNA hydrolysis are described in Bashkin *et al.* (1995), *Appl. Biochem. Biotechnol.* 54:43-56.

Of particular interest in many embodiments are agents that selectively modulate ACAT-2 activity, i.e. agents that modulate the activity of ACAT-2 more
15 than the activity of ACAT-1. In many embodiments, of interest are agents that modulate ACAT-2 activity with substantially no effect, including no effect, on ACAT-1 activity. Of particularly interest in many embodiments are agents that modulate by at least reducing, if not substantially inhibiting, ACAT-2 activity.

As mentioned above, an effective amount of the active agent is administered to
20 the host, where "effective amount" means a dosage sufficient to produce a desired result, where the desired result in the desired modulation, *e.g.* enhancement, reduction, of ACAT-2 activity.

In the subject methods, the active agent(s) may be administered to the host using any convenient means capable of resulting in the desired inhibition of ACAT-2
25 activity. Thus, the agent can be incorporated into a variety of formulations for therapeutic administration. More particularly, the agents of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules,
30 powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols.

As such, administration of the agents can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, etc., administration.

In pharmaceutical dosage forms, the agents may be administered in the form of
5 their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the agents can be used alone or in combination with
10 appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if
15 desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The agents can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher
20 aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The agents can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized
25 acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the agents can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository
30 can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more inhibitors. Similarly, unit dosage forms for
5 injection or intravenous administration may comprise the inhibitor(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a
10 predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in
15 the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the
20 public.

Where the agent is a polypeptide, polynucleotide, analog or mimetic thereof, e.g. antisense composition, it may be introduced into tissues or host cells by any number of routes, including viral infection, microinjection, or fusion of vesicles. Jet injection may also be used for intramuscular administration, as described by Furth
25 *et al.* (1992), *Anal Biochem* **205**:365-368. The DNA may be coated onto gold microparticles, and delivered intradermally by a particle bombardment device, or "gene gun" as described in the literature (see, for example, Tang *et al.* (1992), *Nature* **356**:152-154), where gold microprojectiles are coated with the *ACAT-2* DNA, then bombarded into skin cells.

30 Those of skill in the art will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility

of the subject to side effects. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means.

The subject methods find use in the treatment of a variety of different disease conditions involving ACAT-2 activity, including both insufficient or hypo-ACAT-2 activity and hyper-ACAT-2 activity. Of particular interest is the use of the subject invention to treat patients suffering from disease conditions associated with hyper-ACAT-2 activity, such as disease conditions associated with the presence of elevated cholesterol ester levels, e.g. hypercholesterolemia or hyperlipidemia, including hypertriglyceridemia. In other words, of interest is the use of the subject invention to treat patients suffering from a disease condition where it is desirable to reduced ACAT-2 activity. In many embodiments, the disease conditions will be those in which it is desired to selectively inhibit ACAT-2 activity and not ACAT-1 activity. Other disease conditions include atherosclerosis. The subject compositions may also be used to modulate the immune response in a host, e.g. the immune response of macrophages, where conditions in which such modulation is desirable include immune related diseases, e.g. rheumatoid arthritis, multiple sclerosis, as well as infectious diseases, e.g. toxoplasmosis.

By treatment is meant at least an amelioration of the symptoms associated with the pathological condition afflicting the host, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. symptom, associated with the pathological condition being treated, such as hypercholesterolemia, etc. As such, treatment also includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited, e.g. prevented from happening, or stopped, e.g. terminated, such that the host no longer suffers from the pathological condition, or at least the symptoms that characterize the pathological condition.

A variety of hosts are treatable according to the subject methods. Generally such hosts are "mammals" or "mammalian," where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore (e.g., dogs and cats), rodentia (e.g., mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys). In many embodiments, the hosts

will be humans.

Kits with unit doses of the active agent, usually in oral or injectable doses, are provided. In such kits, in addition to the containers containing the unit doses will be an informational package insert describing the use and attendant benefits of the drugs in
5 treating pathological condition of interest. Preferred compounds and unit doses are those described herein above.

The following examples are offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the formulations, dosages,
10 methods of administration, and other parameters of this invention may be further modified or substituted in various ways without departing from the spirit and scope of the invention.

EXPERIMENTAL

15

I. Cloning of ACAT-2 cDNA

An EST (accession #R10292) was identified by BLAST searches
(<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast>) of the human EST
database with sequences from ACAT-1. Degenerate primers derived from the EST
20 sequences were subsequently used to amplify a partial ACAT-2 cDNA from mouse liver cDNA. Sequences for the 5' and 3' ends of the ACAT-2 cDNA were determined by 5' and 3' rapid amplification of cDNA ends (RACE™) methodology using a mouse spleen Marathon Ready™ cDNA library (Clontech, Palo Alto, CA). ACAT-2
sequences have been deposited in GenBank (accession number AF078751)

25 The mouse ACAT-2 cDNA is predicted to encode a 525-amino acid protein, see SEQ ID NO:03, that is 44% identical to mouse ACAT-1 as reported in Uelmen et al., J. Biol. Chem. (1995) 270:26192-26201, where sequence identity was determined using the GCG algorithm and standard settings. The ACAT-2 protein has multiple hydrophobic domains, and its hydrophobicity plot is strikingly similar to that for
30 ACAT-1. The analysis of mouse ACAT-2 sequences with a transmembrane prediction program (http://ulrec3.unil.ch/software/TMPRED_form.html) indicated that ACAT-2

may have eight transmembrane-spanning regions (amino acid residues 125-143, 154-181, 192-220, 265-285, 304-326, 351-369, 439-459, and 475-496). Using the same program, mouse ACAT-1 was predicted to have seven or eight transmembrane regions. The alignment of the mouse ACAT-2 protein sequence with those of other
5 ACAT gene family members revealed many conserved residues, especially in the C-termini of these proteins. Each family member has a potential tyrosine phosphorylation site and a conserved serine residue (amino acid 248 of ACAT-2) that has been implicated in the catalytic activity of hamster ACAT-1.

10 II. Generation of Recombinant Baculoviruses

Mouse ACAT-2 and human ACAT-1 baculovirus transfer vectors were constructed by inserting ACAT-2 coding sequences (amplified from mouse liver cDNA) or ACAT-1 coding sequences (amplified from HepG2 cell cDNA) downstream of the polyhedrin promoter of the pVL1392 vector (PharMingen, San Diego, CA). Vectors were
15 cotransfected with viral baculogold DNA into Sf9 insect cells, according to the manufacture's protocol (PharMingen Kit), to generate recombinant baculoviruses. Viruses were plaque-purified and high titers of recombinant baculoviruses were generated by two rounds of amplification in Sf9 cells. Cells were cultured at 27 °C in Grace's medium (Life Technologies, Gaithersburg, MD), supplemented with 10%
20 fetal bovine serum.

III. Expression Studies

For protein expression studies, H5 insect cells were cultured in optimized serum-free Express-Five medium (Life Technologies), supplemented with 20 mM
25 L-glutamine. Cells were plated on day 0 at 8.5×10^6 cells per 100-mm dish and infected on day 1 with high-titer baculovirus stocks at an optimal multiplicity of infection (MOI) that was determined empirically. On day 3 (48 h after infection), cells were collected by low-speed centrifugation and washed twice with phosphate-buffered saline. Cell pellets were resuspended in 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4 ,
30 0.03 M EDTA (pH 7.2) and homogenized by 10 passages through a 27-G needle. Cell homogenates were fractionated by centrifugation at $100,000 \times g$ for 1 h at 4°C. The

cell membrane fraction (pellet) was re-suspended in the isolation buffer, and aliquots were frozen (-80°C) for activity assays. Protein concentrations were determined using a DC Protein Assay kit (Biorad, Chicago, IL).

5 IV. Metabolic Labeling

For metabolic labeling, H5 insect cells as prepared above were plated on day 0 at 2.9×10^6 cells per 60-mm dish and infected at day 1 with high-titer baculoviruses at empirically determined MOIs. On day 3 (48 h after infection), cells were washed and incubated in methionine-free and cysteine-free medium (SF900 II, Life Technologies) for 1 h. The medium was then replaced by 3 ml of methionine-free and cysteine-free medium containing 715 μCi of [^{35}S]methionine and [^{35}S]cysteine (Amersham Pro-Mix, Amersham, Arlington Heights, IL) and the cells were incubated for 2 h. Cells were washed twice with phosphate-buffered saline, collected by low-speed centrifugation, resuspended in 500 μl of lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 (pH 7.4)], and sonicated. Cell lysate samples (100 μg protein) were analyzed by 10% SDS-PAGE. Gels were fixed in 30% acetic acid, washed in 25% ethanol and 8% acetic acid, dried, and exposed to x-ray film.

Metabolic labeling of cellular proteins in insect cells 48 h after infection demonstrated the expression of a ~46-kDa protein. The apparent molecular mass of ACAT-2 on SDS-polyacrylamide gels was smaller than that predicted from the amino acid sequence (60.7 kDa), a finding similar to that reported for ACAT-1. See Chang et al., J. Biol. Chem. (1993) 268: 20747-20755.

25 V. ACAT Activity Assays

The rate of incorporation of [^{14}C]oleoyl-CoA (Amersham) into cholesterol esters was assayed essentially as described by Erickson *et al.* J. Lipid Res. (1980)21:930-941. Reactions were performed at 37 °C for 5 min with 100 μg of cell membrane protein, 25 μM oleoyl CoA (specific activity = 18 $\mu\text{Ci}/\mu\text{mol}$), and exogenous cholesterol (20 nmol) added as phosphatidyl choline (PC):cholesterol (4:1 molar ratio) liposomes.

Cell membrane preparations from insect cells infected with the ACAT-2 baculovirus were characterized by high levels of cholesterol esterification activity. The amount of ACAT activity present in ACAT-2-infected cells was consistently higher than that in cells infected with virus containing ACAT-1. However, metabolic labeling studies indicated that considerably more ACAT-2 protein was expressed than ACAT-1. Membrane cholesterol esterification activity was dependent upon the amount of ACAT-2 protein expressed, as demonstrated by a time course of ACAT-2 virus infection

10 VI. Determination of Fatty Acyl CoA Specificity

ACAT activity was assayed essentially as described in Smith et al., J. Lipid Res. (1995) 36:641-652, except that unlabeled fatty acyl CoAs and [^{14}C]cholesterol (51.3 mCi/mmol, NEN DuPont, Boston, MA) provided as cholesterol:egg PC liposomes (molar ratio = 0.2) were used. Parallel assays were performed with [^{14}C]oleoyl CoA (57 mCi/mmol, Amersham) and unlabeled cholesterol provided as PC liposomes (molar ratio = 0.2). The relative competition of fatty acyl CoAs for cholesterol esterification was assessed using [^{14}C]oleoyl CoA diluted to ~20,000 dpm/nmol with unlabeled fatty acyl CoAs (~1:6, mole:mole dilution) and unlabeled cholesterol:PC liposomes (molar ratio = 0.7). The abilities of [^{14}C]oleoyl CoA and [^{14}C]palmitoyl CoA (55.0 mCi/mmol, Amersham) to catalyze cholesterol esterification were assessed directly. Fatty acyl CoAs, egg PC, and cholesterol were from Sigma (St. Louis, MO). Briefly, membrane proteins (25 μg) were incubated in 0.25 M sucrose, 1 mM EDTA, 100 mM Tris-HCl (pH 7.5) containing 400 μg bovine serum albumin and cholesterol:PC liposomes (final volume = 0.2 ml). The reaction was started by the addition of 5 nmol fatty acyl CoA and carried out for 2 min at 37 $^{\circ}\text{C}$. The reaction was terminated by the addition of CHCl_3 :methanol (2:1, v:v), and the products analyzed as described Erickson, *supra*. All assays were in the linear range with respect to assay time and protein concentration.

The ability to express ACAT-1 or ACAT-2 independently in insect cells enabled us to examine several biochemical properties of the enzymes. The esterification of cholesterol by both ACAT-1 and ACAT-2 was dependent on the

presence of a fatty acyl CoA substrate; in its absence, cholesterol esterification activity greater than background was not observed (data not shown). Of the fatty acyl CoAs tested, ACAT-1 showed a slight preference for oleoyl, while that for ACAT-2 was palmitoyl \geq linoleoyl \geq oleoyl \geq arachidonyl. The ability of unlabeled fatty acyl CoAs to compete with [14 C]oleoyl CoA for incorporation into cholesterol esters by ACAT-1 or ACAT-2 was also examined. For ACAT-1, palmitoyl, linoleoyl, and to a lesser extent, arachidonyl competed with oleoyl for incorporation into cholesterol esters. For ACAT-2, both linoleoyl and palmitoyl competed with oleoyl for incorporation into cholesterol esters, but arachidonyl did not. When cholesterol esterification was measured using [14 C]oleoyl CoA or [14 C]palmitoyl CoA directly, both ACAT-1 and ACAT-2 exhibited slightly higher activity with oleoyl CoA.

VII. Determination of Acyl Acceptor Substrate Specificity

The ACAT assay described above was used, except that acceptors other than cholesterol were added to the incubations as acceptor:PC liposomes (molar ratio = ~ 0.2) or, in the case of ethanol, added directly. The acceptors included ethanol, retinol, tocopherol, β -sitosterol, lanosterol, vitamins D1 and D2, diacylglycerol, and a number of oxysterols (25-hydroxy-, 27-hydroxy-, 7 α -hydroxy-, 7 β -hydroxy, 7-keto-, 24(S)-hydroxy-, 24(R)-hydroxy-, 24(S),25-epoxy-, and 24(R),25-epoxycholesterols). The sterols were assessed at three different concentrations (4, 8, and 16 μ g) at a constant sterol:PC molar ratio (~ 0.2). Acceptors were from Sigma or Steraloids (Wilton, NH). 24(S)-hydroxy- and 24(R)-hydroxycholesterol (Prasad et al., J. Steroid Biochem (1984) 21: 733-736, Saucier et al., J. Biol. Chem. (1989) 264: 6863-6869), 24(S),25-epoxy-, and 24(R),25-epoxycholesterols were prepared as described (Saucier, *supra*; Nelson et al., J. Biol. Chem. (1981) 256: 1067-1068; Nelson et al., J. Am. Chem. Soc. (1981) 103:6974-6975). The reaction products were extracted and separated by thin-layer chromatography as described in Erickson, *supra*, and the plates exposed to x-ray film for 4 days to visualize the radioactive spots. Fatty acyl ester standards were used to identify the migration positions of the products. The spots were cut out and counted in a liquid scintillation counter as

described in Erickson, *supra*.

Esterification activity was not detected for either ACAT-1 or ACAT-2 with ethanol, retinol, tocopherol, β -sitosterol, lanosterol, vitamins D1 and D2, or diacylglycerol as acceptors. However, in addition to esterifying cholesterol, both
5 ACAT-1 and ACAT-2 esterified the 3β -hydroxyl group of a variety of oxysterols. All oxysterols analyzed exhibited greater esterification capability than cholesterol itself, with the exception of 7β -hydroxycholesterol for ACAT-1 and 25-hydroxycholesterol for ACAT-2. For ACAT-1, the highest levels of incorporation into sterol esters were for 24(R)-hydroxycholesterol, 7α -hydroxycholesterol, and 27-
10 hydroxycholesterol. For ACAT-2, 24(R)-hydroxycholesterol, 27-hydroxycholesterol, and 7β -hydroxycholesterol were esterified at the highest rates.

The effects of added oxysterols on membrane cholesterol esterification was also examined, using the small but significant mass of endogenous membrane cholesterol as a substrate. For ACAT-1, cholesterol esterification was increased by 27-
15 hydroxycholesterol and 24(S),25-epoxycholesterol and was decreased substantially by 7α -hydroxycholesterol, 25-hydroxycholesterol, and 24(S)-hydroxycholesterol. For ACAT-2, none of the oxysterols increased cholesterol esterification; instead, they either had little effect or decreased it by 40-60%.

20 VIII. Inhibitor Studies

Cholesterol esterification activity was assayed as described above [using [14 C]oleoyl CoA and cholesterol:PC liposomes (molar ratio = 0.2)] after a 5-min preincubation of the membranes at 37 °C with 1 mM PMSF, 0.1 mM para-hydroxymercuribenzoate (PHMB), or 0.1 mM progesterone. Inhibitors were from
25 Sigma.

The specific ACAT inhibitors PD 132301-2 [N-[2,6-bis(1-methylethyl)-phenyl]-N'-[[1-[4-(dimethyl-amino) phenyl]cyclopentyl]methyl]urea, hydrochloride], CI-976 [2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide] and CI-1011 [[(2,4,6-triisopropyl-phenyl)-acetyl]-sulfamic acid 2,6-diisopropyl-pher] were
30 provided by Parke-Davis Pharmaceuticals (Ann Arbor, MI). Stock solutions (20 mM) of the inhibitors were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C.

Membrane proteins (100 µg for ACAT-1 and 50 µg for ACAT-2) were pre-incubated with cholesterol:PC liposomes and different inhibitors (final DMSO concentration = 2.5%) for 30 min on ice. After equilibrating for 5 min at 37 °C, the reaction was started by adding [¹⁴C]oleoyl CoA. Assays were performed otherwise essentially as described above.

The effects of nonspecific (PMSF, PHMB, and progesterone) and specific inhibitors of ACAT were examined next. PMSF, which inhibits enzymes utilizing serine in their active site, inhibited both ACAT-1 and ACAT-2 by 20-40%. PHMB, which acts on enzymes that require sulfhydryl groups for activity, inhibited ACAT-1 to a greater extent than ACAT-2, and progesterone, a compound known to inhibit ACAT, inhibited both enzymes equally. Membranes from ACAT-1 and ACAT-2 virus-infected cells were also assayed in the presence of ACAT-specific inhibitors PD 132301-2, CI-976, and CI-1011. PD 132301-2 inhibited both ACAT-1 and ACAT-2 to similar degrees. In contrast, CI-976 exhibited some selectivity for the expressed enzymes. ACAT-1 activity was suppressed by CI-976 with an apparent IC₅₀ of ~5 µM, whereas ACAT-2 activity was more sensitive with an apparent IC₅₀ of < 1 µM. A significant difference in selectivity was also observed for CI-1011, which also inhibited ACAT-2 (IC₅₀ ~2.5 µM) more than ACAT-1 (IC₅₀ > 10 µM).

IX. ACAT-2 Expression in Mouse and Human Tissues

For RT-PCR analysis, total RNA was prepared from fresh mouse tissues, human hepatoma cells (HepG2), human small intestine, or human fibroblasts using Trizol (Life Technologies), and cDNA was synthesized using random hexamer primers and Superscript reverse transcriptase (Life Technologies). For mouse ACAT-2, a 531-bp fragment was amplified using sense (5' ACTGTGCCTGGGATCTTTTGTGTC 3')(SEQ ID NO:05) and antisense (5' CTCGCGGGGTGGCCATGCTGGGAGTG 3') (SEQ ID NO:06) primers. For human ACAT-2, a 742-bp fragment was amplified using sense (5' TCTTCTATCCCGTCATGCTG 3') (SEQ ID NO:07) and antisense (5' GGTCCACATCAGCACGTTCC 3') (SEQ ID NO:08) primers. Amplification of

glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (an internal control) was as described in Farese et al., J. Lipid Res. (1996) 37:347-360.

For northern blots, total RNA was extracted from tissues using Trizol (Life Technologies) and 10- μ g aliquots were analyzed. Blots were hybridized with a 32 P-labeled 1.5-kb DNA fragment containing the mouse ACAT-2 coding sequences and re-probed with coding sequences for G3PDH as an internal standard.

ACAT-2 mRNA was detected in a variety of mouse tissues by RT-PCR with the highest levels of expression in the liver, small intestine, and embryo liver. Northern analysis of mouse tissues demonstrated a ~2-kb ACAT-2 mRNA in the liver and small intestine. To determine if ACAT-2 was also expressed in the liver and intestine in humans, RT-PCR experiments on cDNA from HepG2 cells (a human hepatoma cell line), human intestine, and human fibroblasts were performed. ACAT-2 mRNA was detected in HepG2 cells and small intestine, but not in fibroblasts. In a separate experiment, we also detected ACAT-2 mRNA in cDNA from human liver.

15

X. Mapping of Mouse *Acact*-2 by Linkage Analysis

Linkage analysis was performed using a panel of 67 progeny derived from a ((C57BL/6J x *Mus spretus*)F1 X C57BL/6J) interspecific backcross as described in Warden et al., Genomics (1993) 18:295-307. This backcross panel has been typed for more than 400 loci distributed throughout the genome as described in Welch et al, J. Lipid Res. (1996)37:1406-1421. Briefly, parental strain DNAs were screened for restriction fragment-length variants following digestion of genomic DNA with a panel of 10 restriction enzymes and hybridization with a fluorescein-labeled (Gene Images random prime labeling module, Amersham), 1.3-kb human *Acact2* cDNA fragment. Filters were washed under conditions of high stringency (0.1 x SSC/0.1% SDS, 60 °C, 20 min). *Acact2* hybridization products were detected by chemiluminescence (Gene Images CDP-Star detection module, Amersham). Linkage to previously typed chromosomal markers was detected using Map Manager v2.6.5, and loci were ordered by minimizing the number of recombination events between *Acact2* and the markers as described in Manly, Mamm. Genome (1993) 4:303-313. The data have been deposited in the Mouse Genome Database under accession

30

number MGD-J:45730 (<http://www.infomatics.jax.org>).

The mouse ACAT-2 gene (*Acact2*) was localized to mouse chromosome 15 by linkage analysis from an interspecific backcross. The hybridization of *Bam*HI-digested DNA of the parental strains with the *Acact2* cDNA probe resulted in the
5 detection of single fragments in DNA from each (C57BL/6J = 10 kb and *M. spretus* = 8.8 kb) and both fragments in DNA from the F1 progeny. The segregation pattern of the *M. spretus* allele among the backcrossed mice revealed linkage to markers on chromosome 15; linkage was not detected with markers on any other chromosome. No recombination was observed between *Acact2* and the microsatellite marker
10 *D15Mit16* (0/65 mice). The following gene order was obtained (distance \pm SE, in centiMorgans): centromere-*D15Mit31*-(3.6 \pm 2.5 cM)-*Ppara*-(9.2 \pm 3.6)-*D15Ucla3*, *Pou6f1*-(1.5 \pm 1.5)-*Acact2*, *D15Mit16*. A human EST for *Acact2* (accession #R10292) has been mapped to a homologous region on human chromosome 12q13.3-q15 (<http://www.ncbi.nlm.nih.gov/XREFdb>).

15

XI. ACAT-2 Knockout Studies

A. Generation of ACAT-2 Knockout Mice

To generate ACAT-2 knockout mice, a gene targeting vector was constructed
20 so that a *neo* gene would replace the C-terminal sequences of the mouse ACAT-2 gene. This vector was electroporated into RF-8 embryonic stem cells (strain 129/SvJae) and targeted clones were identified. One of these clones was used for microinjections of mouse blastocysts to generate chimeric offspring. High percentage chimeras were bred with C57BL/6 mice to transmit the targeted mutation through the
25 germline. Mice heterozygous for the ACAT-2 knockout mutation were intercrossed to generate homozygotes, which were viable and present in the expected Mendelian distribution. Analysis of liver RNA by RT-PCR demonstrated loss of the C-terminal sequences of the ACAT-2 mRNA, confirming that the targeted mutation disrupted the gene.

30

B. Assays

1. Hepatic ACAT activity and hepatic cholesterol content.

Hepatic ACAT activity was measured in mice fed a chow diet. We found ~35% residual ACAT activity in mice heterozygous for the mutant allele (n=3), and less than 1% residual activity in homozygous ACAT-2 knockout mice (n=6) compared with wild-type control mice (n=6).

Hepatic free cholesterol content was similar in wild-type and ACAT-2-deficient mice (1.96 ± 0.03 vs 1.97 ± 0.08 mg/g tissue, p=NS) but hepatic cholesterol ester content was significantly reduced in knockout mice (0.57 ± 0.12 vs 0.07 ± 0.004 mg/g tissue, $p < 0.06$) (n=6 in each group).

2. Lipoprotein Analysis

Plasma total cholesterol, plasma free cholesterol percentage, and plasma triglycerides were measured in mice fed a chow diet. No significant differences were found between the two groups:

Plasma total cholesterol:	WT 85.31 ± 13.48
	KO 72.75 ± 21.22 , p=NS, by Mann-Whitney
Free cholesterol %:	WT 26.15 ± 0.97
	KO 26.67 ± 1.19 , p=NS by Mann-Whitney
Plasma Triglycerides:	WT 31.79 ± 2.8
	KO 40.52 ± 3.23 , p=NS by Mann-Whitney

In addition, the lipoprotein profile by fast performance liquid chromatography (FPLC) was similar for wild-type and knockout mice.

In contrast, a significant difference between wild-type and knockout mice was observed when the mice were challenged with a diet rich in cholesterol and cholic acid ("Paigen" diet). Under this condition, plasma total cholesterol increased more than 2-fold in wild-type mice, but remained at basal level in knockout mice (202.9 ± 11.6 vs 69.52 ± 5.4 , $p < 0.06$). FPLC analysis revealed that this blunted response to diet-induced hypercholesterolemia in knockout mice is due to the absence of VLDL-

cholesterol. It is likely that the absence of plasma VLDL cholesterol in knockout mice fed the Paigen diet is due to impaired hepatic output of VLDL cholesterol into the blood stream (rather than increased clearance of plasma VLDL), but this remains to be determined.

5

3. Intestinal cholesterol absorption

Intestinal cholesterol absorption was measured by the dual isotope method in mice fed a chow diet. No significant difference was found between wild-type and knockout mice (n=6 in each group) in percent cholesterol absorption (22.9 ± 3.5 vs.

10 22.3 ± 4.1 , p=NS) or fecal neutral sterol excretion (22.3 ± 1.6 vs 20.1 ± 1.4 $\mu\text{mol/day/100g}$ body weight, p=NS).

Bile acid pool size was also measured and was found to be larger in wild-type than in knockout mice (69.24 ± 4.56 vs 56.69 ± 3.94 $\mu\text{mol/100g}$ body weight) but this experiment will need to be repeated for confirmation. Bile acid pool composition was

15 not significantly different between the two groups.

Intestinal cholesterol content in these mice was also measured. While the intestinal total cholesterol content is comparable between wild type and knockout mice (3.72 ± 0.11 vs. $4.03 \pm .24$ mg/g tissue, p=NS), the amount of esterified cholesterol

20 was significantly higher in wildtype mice compared with knockout (0.75 ± 0.06 vs. 0.12 ± 0.01 , $p < 0.06$).

C. Discussion of Results

The above results indicate that cholesterol esterification by ACAT-2
25 could plays a major role in intestinal cholesterol absorption (for example, under dietary conditions of high cholesterol intake), and in the synthesis of lipoproteins by the intestine (chylomicrons) and the liver (VLDL, which are metabolized to LDL). Thus, ACAT-2 is an important determinant of plasma cholesterol levels.

30 As seen in the above results, ACAT-2 knockout mice are viable, fertile, and healthy. They have a near complete lack of ACAT activity in the liver, establishing

ACAT-2 as the major ACAT in this organ. Additionally, cholesterol ester levels are markedly reduced in both the liver and the intestine. ACAT-2 knockout mice have normal plasma cholesterol levels on a low-fat, low-cholesterol diet. However, whereas wild-type mice have a marked increase in plasma cholesterol when fed a synthetic diet
5 rich in fat and cholesterol, the ACAT-2 knockout mice do not. This establishes that ACAT-2 expression is an important determinant of the plasma cholesterol response to dietary intake in mice. Intestinal cholesterol absorption is normal in ACAT-2 knockout mice on a low-cholesterol diet.

The preliminary data from ACAT-2 knockout mice provide clear rationale that
10 a selective inhibitor of ACAT-2 in humans is a useful strategy to lower plasma cholesterol levels. This is the case for fasting cholesterol levels or for lowering the response of plasma cholesterol to dietary cholesterol intake. Inhibitors developed to date have been targeted against ACAT activity in general and are unlikely to be selective (e.g.,
15 see J. Biol. Chem. (1998) 273:26755-26764).

It is apparent from the above results and discussion that polynucleotides encoding novel mammalian ACAT-2 enzymes, as well as the novel polypeptides encoded thereby, are provided. The subject invention is important for both research
20 and therapeutic applications. Using the ACAT-2 probes of the subject invention, the role of ACAT-2 and its regulation in a number of physiological processes can be studied *in vivo*. The subject invention also provides for important new ways of treating diseases associated with ACAT-2 activity.

25 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication
30 by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the
5 spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A polynucleotide present in other than its natural environment encoding an ACAT-2 polypeptide.
- 5 2. A fragment of a polynucleotide according to Claim 1.
3. An ACAT-2 polypeptide present in other than its naturally occurring environment.
- 10 4. A fragment of a polypeptide according to Claim 4.
5. An expression cassette comprising a transcriptional initiation region functional in an expression host, a polynucleotide having a nucleotide sequence found in the
- 15 nucleic acid according to Claim 1 under the transcriptional regulation of said transcriptional initiation region, and a transcriptional termination region functional in said expression host.
6. A cell comprising an expression cassette according to Claim 9 as part of an
- 20 extrachromosomal element or integrated into the genome of a host cell as a result of introduction of said expression cassette into said host cell, or the cellular progeny thereof.
7. A method of producing an ACAT-2 polypeptide, said method comprising:
- 25 growing a cell according to Claim 6, whereby said polypeptide is expressed; and
- isolating said polypeptide substantially free of other proteins.
8. A monoclonal antibody binding specifically to ACAT-2.
- 30 9. A method for inhibiting the activity of ACAT-2, said method comprising:

contacting said ACAT-2 with an agent that inhibits the activity of said ACAT-

2.

10. A method of modulating a symptom in a mammalian host of a disease

5 condition associated with ACAT-2 activity, said method comprising:

administering to said host a pharmaceutical composition comprising an
effective amount of an active agent that modulates said ACAT-2 activity in said host.

11. The method according to Claim 10, wherein said agent inhibits said ACAT-2

10 activity.

12. The method according to Claim 11, wherein said agent selectively inhibits said
ACAT-2 activity.

SEQUENCE LISTING

<110> Cases, Sylvaine
 Farese, Robert
 Novak, Sabine
 Erickson, Sandra
 Allard, Michel

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13683

A. CLASSIFICATION F SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/15, 193, 252.3, 320.1; 536/23.2, 23.5; 530/350; 424/142.1, 158.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/45439 A1 (TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 04 December 1997. See abstract, examples 1-5 and claims.	1-7, 9-12
Y	ROARK, W. H. et al. Inhibitors of Acyl-CoA:Cholesterol Acyltransferase (ACAT). 2. Modification of Fatty Acid Anilide ACAT Inhibitors: Bisosteric Replacement of the Amide Bond. J. Med. Chem. 28 May 1993, Vol. 36, No. 11, pages 1662-1668. See the entire article.	9-12
X, P	OELKERS, P. et al. Characterization of Two Human Genes Encoding Acyl Coenzyme A: Cholesterol Acyltransferase-related Enzymes. J. Biol. Chem. 09 October 1998, Vol. 273, No. 41, pages 26765-26771, especially page 26767.	1-7

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

03 SEPTEMBER 1999

Date of mailing of the international search report

21 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

TEKCHAND SAIDHA

Telephone No. (703) 308-0196

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13683

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6A(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13683

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 9/10, 1/20, 15/00; C07K 14/00; C07H 21/04; C12Q 1/48; A61K 39/395.

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/15, 193, 252.3, 320.1; 536/23.2, 23.5; 530/350; 424/142.1, 158.1.

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; STN files included medline, caplas, biosis, scisearch, biotechd, embase; search terms : acyl-CoA cholesterol acyltransferase 2 (or II), ACAT-2 or ACAT II; ACAT and (polynucleotide or dna or gene or ma).

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-2 & 5-7, drawn to polynucleotide encoding acyl-CoA : cholesterol acyltransferase 2 (ACAT-2).

Group II, claim(s) 3-4, drawn to ACAT-2 polypeptide.

Group III, claim(s) 8, drawn to antibody to ACAT-2 polypeptide.

Group IV, claims 9-12, drawn to method of inhibiting or modulating the activity of ACAT-2.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Prior art reveals the polynucleotide encoding the ACAT-2 [WO 97/454439, 4 December(1997)]. With the polynucleotide and the encoded ACAT-2 protein known, the invention of Groups I & II or I & III, I & IV lack the corresponding special technical feature and therefore lack the Unity of Invention. For the same reasons the inventions of Groups II & III, II & IV or III & IV lack the corresponding special technical feature and therefore lack the Unity of Invention.